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# Feo, the *Drosophila* Homolog of PRC1, Is Required for Central-Spindle Formation and Cytokinesis

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## Summary

**We performed a functional analysis of *fascetto (feo)*, a *Drosophila* gene that encodes a protein homologous to the Ase1p/PRC1/MAP65 conserved family of microtubule-associated proteins (MAPs) [1–5]. These MAPs are enriched at the spindle midzone in yeast and mammals and at the fragmoplast in plants, and are essential for the organization and function of these microtubule arrays [1–5]. Here we show that the Feo protein is specifically enriched at the central-spindle midzone and that its depletion either by mutation or by RNAi results in aberrant central spindles. In Feo-depleted cells, late anaphases showed normal overlap of the antiparallel MTs at the cell equator, but telophases displayed thin MT bundles of uniform width instead of robust hourglass-shaped central spindles. These thin central spindles exhibited diffuse localizations of both the Pav and Asp proteins, suggesting that these spindles comprise improperly oriented MTs. Feo-depleted cells also displayed defects in the contractile apparatus that correlated with those in the central spindle; late anaphase cells formed regular contractile structures, but these structures did not constrict during telophase, leading to failures in cytokinesis. The phenotype of Feo-depleted telophases suggests that Feo interacts with the plus ends of central spindle MTs so as to maintain their precise interdigitation during anaphase-telophase MT elongation and antiparallel sliding.**

## Results and Discussion

When the cleavage furrow begins to form during late anaphase and telophase, the spindle microtubules (MTs) of animal cells reorganize to form a robust network of antiparallel MTs in the region between the separating sets of chromosomes. The plus ends of these antiparallel MTs interdigitate at the center of the cell, and their minus ends terminate near the chromosomes [6, 7]. We refer to this ana/telophase MT network as the "central spindle" and to its middle part as the "central-spindle midzone." A large body of data indicates that the central spindle is essential for cytokinesis [8, 9]. However, the mechanisms underlying the formation and dynamics of the central spindle remain poorly understood.

Here we show that *feo*, the *Drosophila* homolog of human *PRC1*, is required for central-spindle formation and cytokinesis. *feo* is identified by several alleles, including the $feo^{EA86}$ and $feo^{S27}$ mutations induced by ethylmethanesulfonate and by P element mobilization, respectively (FlyBase: http://flybase.bio.indiana.edu/). Cytological analysis of squashed brain preparations from third-instar larvae showed that these two mutations result in high proportions (46%–77%) of polyploid cells and normal frequencies of anaphases (Table 1). These phenotypes are very similar to those elicited by other mutations that disrupt neuroblast cytokinesis [10–13], and they suggest that the wild-type function of *feo* is required for this process.

To further characterize the *feo* phenotype, we stained brain preparations from $feo^{EA86}$ and $feo^{S27}$ mutant males for tubulin, myosin II, and DNA. *Drosophila* brains contain mostly two types of dividing cells: neuroblasts (NBs) and ganglion mother cells (GMCs). NBs are stem cells that divide asymmetrically to produce another NB and a smaller GMC; GMCs divide symmetrically only once and give rise to two neurons or glial cells [14, 15]. Brain preparations from $feo^{EA86}$ and $feo^{S27}$ mutant males did not show defects in the early stages of NB and GMC divisions. Cells in both types of mutants displayed regular asters and metaphase figures, and they entered anaphase normally. Middle and late anaphases in mutants also had a normal appearance and exhibited an apparently regular overlapping of antiparallel MTs at the center of the cell (Figure 1A). However, mutant telophases, recognized by the morphology of chromosomes and asters, showed severely abnormal central spindles. In early telophases, central spindles appeared less dense than in the wild-type, and in middle and late telophases they consisted of a thin bundle of MTs of homogeneous width instead of displaying the typical hourglass shape of wild-type central spindles (Figure 1A).

We also immunostained *feo* brain cells for Pavarotti (Pav) and Abnormal spindle (Asp), two MT binding proteins required for *Drosophila* cytokinesis [16–18]. Pav is a kinesin-like protein that accumulates at the central spindle midzone of early telophases and remains associated with this region throughout telophase (Figure 1C, [16]). In early telophases of *feo* mutants, Pav staining was comparable to that in the wild-type (data not shown). In mutant late telophases, however, the Pav fluorescent signal was not restricted to the central-spindle midzone as in the wild-type but was spread over a larger region (Figure 1C). Mutant telophases also differed from their wild-type counterparts with regard to the pattern of Asp immunostaining. In wild-type telo-

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Table 1. Frequencies of Polyploid Cells and Anaphase Figures in *feo* Mutant Brains

| Genotype | Number of Cells Scored | Polyploid Cells (Percent) | Anaphases (Percent) | Mitotic Index[a] |
|---|---|---|---|---|
| $feo^{EA86}/Y$ | 724 | 76.7 | 13.4 | 0.40 |
| $feo^{S27}/Y$ | 921 | 45.8 | 14.4 | 0.37 |
| *FM7/Y* (control) | 424 | 0 | 15.0 | 0.60 |
| $feo^{EA86}/feo^{EA86}$ | 414 | 62.3 | 18.6 | 0.24 |
| $feo^{EA86}/Df(1)v\text{-}L3$ | 504 | 65.5 | 14.8 | 0.35 |
| $feo^{S27}/Df(1)v\text{-}L3$ | 678 | 73.7 | 11.9 | 0.30 |

[a]To estimate the mitotic index, we determined the average number of mitotic figures per optic field. The optic field chosen for this analysis is the circular area defined by a 100× phase-contrast Neofluar Zeiss objective lens with 10× oculars with the optovar set at 1.25.

phases, Asp accumulates at both the spindle poles and the extremities of the central spindle, suggesting a preferential binding of this protein to the MT minus ends (Figure 1B; [17, 18]). In contrast, *feo* telophases displayed a regular Asp staining at the spindle poles but did not exhibit any detectable Asp signal at the extremities of their aberrant central spindles (Figure 1C).

In addition to defects in the central spindle, mutant *feo* telophases also exhibited defective contractile rings. In early telophases, contractile rings appeared morphologically normal, but in late telophases, most of these rings remained poorly constricted and were either discontinuous or consisted only of patches of myosin II (Figure 1A). Taken together, the results of our cytological analyses indicate that the wild-type function of *feo* is required for proper assembly and behavior of both the central spindle and the contractile ring. These structures are equally affected in both NBs and GMCs, demonstrating that *feo* is required for cytokinesis in both cell types.

## Molecular Analysis of *feo*

The *feo* gene [*l(1)BP1*] was previously mapped to region 9F, distal to the *vermilion* gene on the *Drosophila* X chromosome [19]. Starting with cloned *vermilion* sequences [20], we conducted a chromosome walk and located the positions of several chromosomal rearrangements that delimited the location of *feo* to a 20 kb region that contains eight predicted genes (Figure 2A). The analysis of $feo^{S27}$ showed that this mutation had a P element inserted into the coding sequence (37 bp downstream the ATG start codon) of the predicted intronless gene *CG11207* (Figure 2A). To verify that *CG11207* is indeed *feo,* we introduced in flies a transgene including the entire *CG11207* coding region. This transgene rescued the lethality and the cytological phenotypes associated with mutations in *feo*.

Further evidence that *CG11207* is *feo* is provided by the molecular analysis of $feo^{EA86}$. The wild-type *feo* gene encodes a putative protein of 672 amino acids. The DNA sequence of $feo^{EA86}$ revealed that this mutant allele contains a 14 bp deletion spanning nucleotides 830–843 of the *CG11207* gene; this deletion leads to a frameshift mutation that introduces a stop codon at position 306 of the amino acid sequence. This results in a truncated protein of 305 amino acids, with an altered sequence from residues 277 to 305 (Figure 2B).

BLAST searches indicated that the Feo protein is a member of a conserved family of spindle midzone proteins, including Ase1p of *S. cerevisiae*, NtMAP65-1 of tobacco, AtMAP65-3/PLE of *Arabidopsis*, and human PRC1 [1–5]. *feo* is also highly homologous to the *Drosophila* gene *CG1655* immediately adjacent to *feo*. This gene, which we name *sister of feo (sofe)*, also lacks introns and encodes a putative protein of 607 amino acids (see FlyBase). *sofe* is transcribed in S2 tissue culture cells and in several tissues throughout development, but it does not appear to have an essential mitotic function (see the Supplemental Data available with this article online). The highest sequence identity among the Feo-related proteins is in a central 41 amino acid region (Figure 2B) that contains a 16 amino acid conserved motif required for Ase1p function in vivo [4]. In addition to this motif, these proteins also share conserved organizations; they all contain coiled-coil regions [4]. Like PRC1, Feo exhibits two Cdk phosphorylation sites, but the location of these sites differs, and Feo lacks the nuclear localization signals that have been described in PRC1 [1, 3].

## Subcellular Location of the Feo Protein

To determine the subcellular localization of Feo, we raised two antibodies against parts of the Feo protein; one was directed to amino acids 1–148, and the other was directed to amino acids 424–672. Western blot analysis showed that both antibodies recognize a band of the expected size ($M_w$ = 78 kDa). This band is reduced in $feo^{EA86}/+$ heterozygous females and is absent in $feo^{EA86}$ hemizygous males (Figure 3A). Immunolocalization experiments with both antibodies revealed that Feo is enriched at the central-spindle midzone of both NBs and GMCs (Figures 3B and 3C). In interphase brain cells, Feo is dispersed in the cytoplasm and is excluded from the nucleus (data not shown). Prometaphases, metaphases, and early anaphases did not exhibit Feo accumulations on the spindle microtubules. A clear Feo signal was seen only in late anaphase and during telophase and was always associated with the central spindle midzone (Figure 3). Immunostaining of mitotic figures from $feo^{EA86}$ mutants did not reveal any signal, confirming the specificity of these antibodies (data not shown).

We also performed Feo immunolocalization experiments on wild-type spermatocytes. Here also, Feo immunostaining was restricted to the MTs of the central-spindle midzone (Figure 3D), suggesting a role of Feo in spermatocyte cytokinesis. However, we have been unable to examine meiosis in *feo* mutant males because their testes are very small and devoid of dividing spermatocytes. The absence of meiotic cells in mutant testes

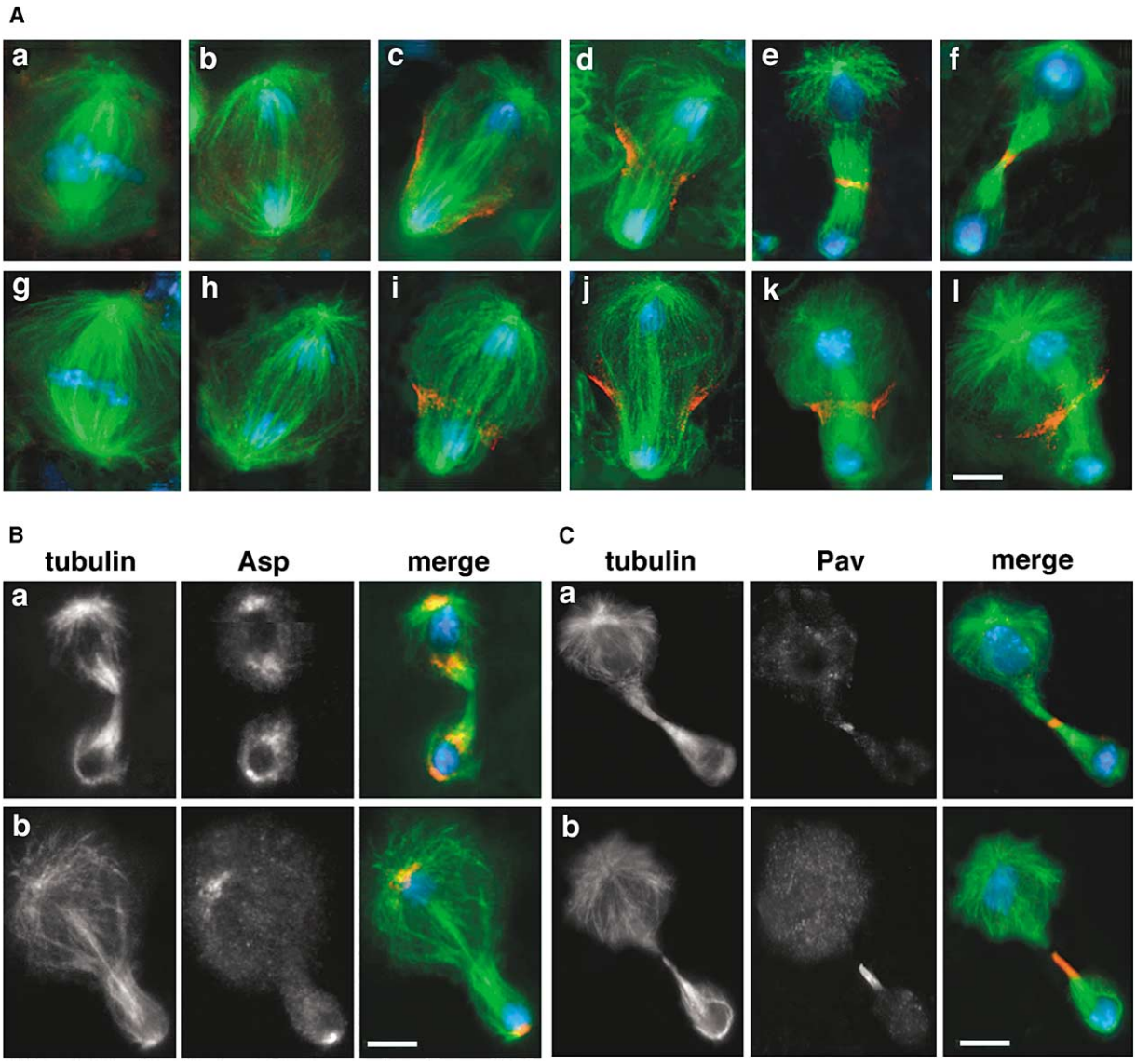


Figure 1. Phenotypic Analysis of *feo* Mutant Neuroblasts

(A) Wild-type (a–f) and *feo* mutant (g–l) neuroblasts stained for tubulin (green), myosin II (orange), and DNA (blue). (a and g) Metaphases; (b and h) early anaphases; (c and i) late anaphases; (d and j) early telophases; (e and k) mid-telophases; and (f and l) late telophases. Note the aberrant structure of the central spindle and the failure of actomyosin ring constriction in *feo* mutant telophases.

(B) Wild-type (a) and *feo* (b) late telophases stained for tubulin (green), Asp (orange), and DNA (blue). Asp signal is absent from the extremities of the mutant central spindle.

(C) Wild-type (a) and *feo* (b) late telophases stained for tubulin (green), Pav (orange), and DNA (blue). Note the broad Pav signal at the central spindle midzone of the *feo* mutant telophase.

The scale bars represent 5 μm.

is a likely consequence of successive failures of cytokinesis during germline proliferation.

### Disruption of *feo* by RNA Interference (RNAi)

To determine the consequences of Feo depletion, we treated S2 tissue culture cells with *feo* double-stranded RNA (dsRNA). RNAi worked quite well; the Feo protein was effectively depleted after the cells were exposed to the dsRNA for 72 hr (Figure 4A) and was not detected in cytological preparations from such RNAi cells (Figure S1 in the Supplemental Data). Examination of these cells with a fluorescence-activated cell sorter (FACS) revealed a higher proportion of 8C polyploids than in controls (Figure 4B). Most Feo-depleted cells were binucleated (Figure 4C), consistent with a defect in cytokinesis [21].

To determine the primary defect leading to failure of cytokinesis in the absence of Feo, we stained *feo* RNAi cells for tubulin and DNA as well as Pav, Asp, or actin. Feo depletion did not affect the spindle morphology of prophase, metaphase, or early anaphase mitotic figures. However, central spindle organization during telophase was disrupted. In the absence of Feo, 90% of telophases

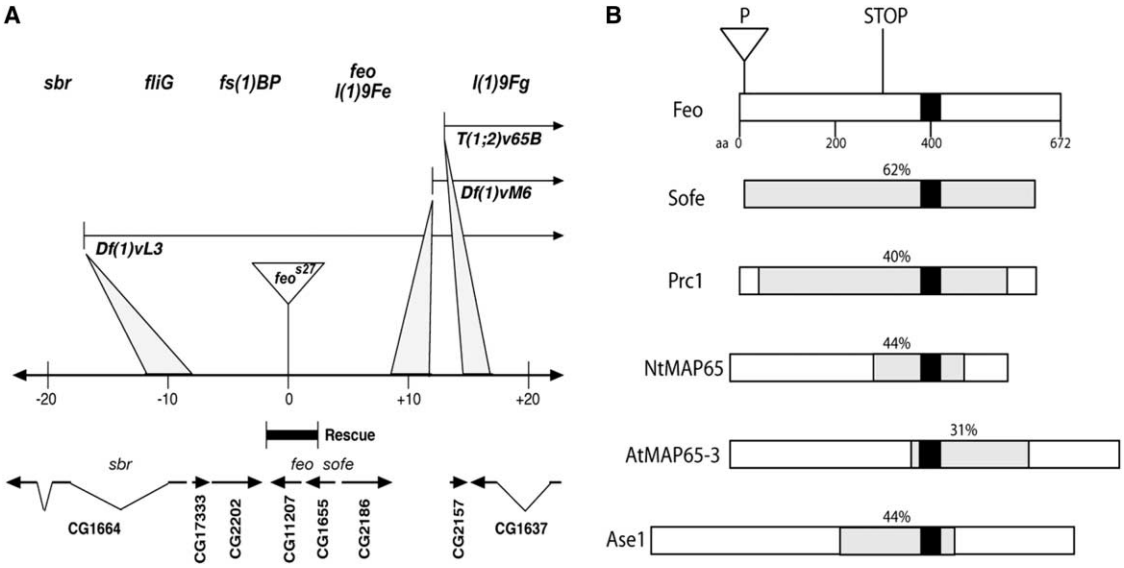


Figure 2. The *feo* Gene and Its Protein Product

(A) The *feo* region on the *Drosophila* X chromosome. Orientation is with the telomere to the left and the centromere to the right. (Top) A genetic map showing the position of *feo* relative to the adjacent genes *short bristles* (*sbr*), *flightless G (fliG)*, *fs(1)BP*, and *l(1)9Fg*, based on data from [19, 22]. Horizontal lines indicate either material removed by the deletions *Df(1)vM6* and *Df(1)vL3* or the X chromosomal DNA transposed to the second chromosome in *Tp(1;2)v65b*. (Bottom) A molecular map of the *feo* region. Distances are in kb, with 0 representing the location of the P element insertion associated with $feo^{S27}$. Gray indicates the region of uncertainty in the molecular coordinates of the three rearrangement breakpoints delimiting *feo*. Arrows show the 5′-to-3′ orientation of transcriptional units annotated by FlyBase (http://flybase.bio.indiana.edu/). Introns smaller than 1 kb are not displayed; neither *feo* nor the adjacent related gene *sofe* contains introns. "Rescue" denotes the fragment of genomic DNA that rescues both the lethality and cytological defects associated with *feo* when it is transformed into *Drosophila*.

(B) Evolutionary conservation of the Feo protein. The triangle and the STOP sign indicate the site of the P element insertion in $feo^{S27}$ and the position of the stop codon in $feo^{EA86}$, respectively. The black segments across all the proteins of the family correspond to a conserved 41 amino acid region identified by ClustalW. This region contains the 16 amino acid conserved motif that has been shown to be essential for Ase1p function in vivo [4]. Gray regions show lower but demonstrable homologies with Feo. The indicated percents of similarity between Feo and the shaded area of each homologous counterpart were determined with BLAST2.

(n = 85) displayed a thin and compact bundle of microtubules between the two daughter nuclei instead of a robust, hourglass-shaped central spindle (compare panels a and b of Figure 4D). We named the gene *fascetto* after this phenotype; fascetto means "thin bundle" in Italian. Notably, cells with aberrant central spindles did not display defects in spindle pole separation; telophases with abnormally thin central spindles were slightly longer (26 $\mu$m $\pm$ 0.81; n = 92) than their control counterparts (23 $\mu$m $\pm$ 0.73; n = 119).

The Pav and Asp proteins were normally localized in late anaphases of *feo* RNAi cells (Figure S2). However, the aberrant central spindles of Feo-depleted telophases showed diffuse localizations of both proteins (Figure 4D). These results are consistent with those obtained with *feo* mutant NBs and GMCs and confirm that Feo is required for proper organization of central spindle MTs.

Feo-depleted S2 cells were also defective in contractile-ring assembly. In normal S2 cells, actin concentrates in a wide equatorial band during late anaphase (Figure S2). As cell division proceeds, this band progressively narrows to form the contractile ring (Figure 4D; see also [21]). In Feo-depleted cells, actin formed a regular late-anaphase wide band (Figure S2), but this band failed to narrow, and a contractile ring did not form (Figure 4D). As a result, there was very limited cleavage furrow ingression, and cytokinesis failed.

## The Role of Feo in *Drosophila* Cytokinesis

Feo shares a high degree of homology with PRC1, and both proteins are required for central-spindle formation and cytokinesis. Moreover, overexpression of either protein results in extensive bundling of interphase MTs ([3]; Figure S4). However, Feo and PRC1 also exhibit several divergent properties. Whereas PRC1 has a nuclear localization signal and accumulates in interphase nuclei [3], Feo lacks this signal and is cytoplasmic. Feo's MT binding ability also appears to differ from that of PRC1. PRC1 binds the MTs of metaphase and early anaphase spindles and then concentrates in the central-spindle midzone [3]. In contrast, Feo does not bind the MTs of early mitotic spindles but specifically accumulates at the central-spindle midzone. Finally, whereas the absence of PRC1 results in partial or complete disruption of the central spindle [3], depletion of Feo does not abolish central-spindle assembly but instead causes the formation of a characteristic, aberrant central spindle.

The features of the aberrant central spindles in Feo-depleted cells strongly suggest that these structures contain improperly oriented MTs. The plus ends of MTs from the opposite half of the spindles overlap at the center of mid-anaphase figures from *feo* mutant neuroblasts and *feo* RNAi cells. However, this is not sufficient for the subsequent formation of a regular central spindle because both cells types form an aberrant central spin-

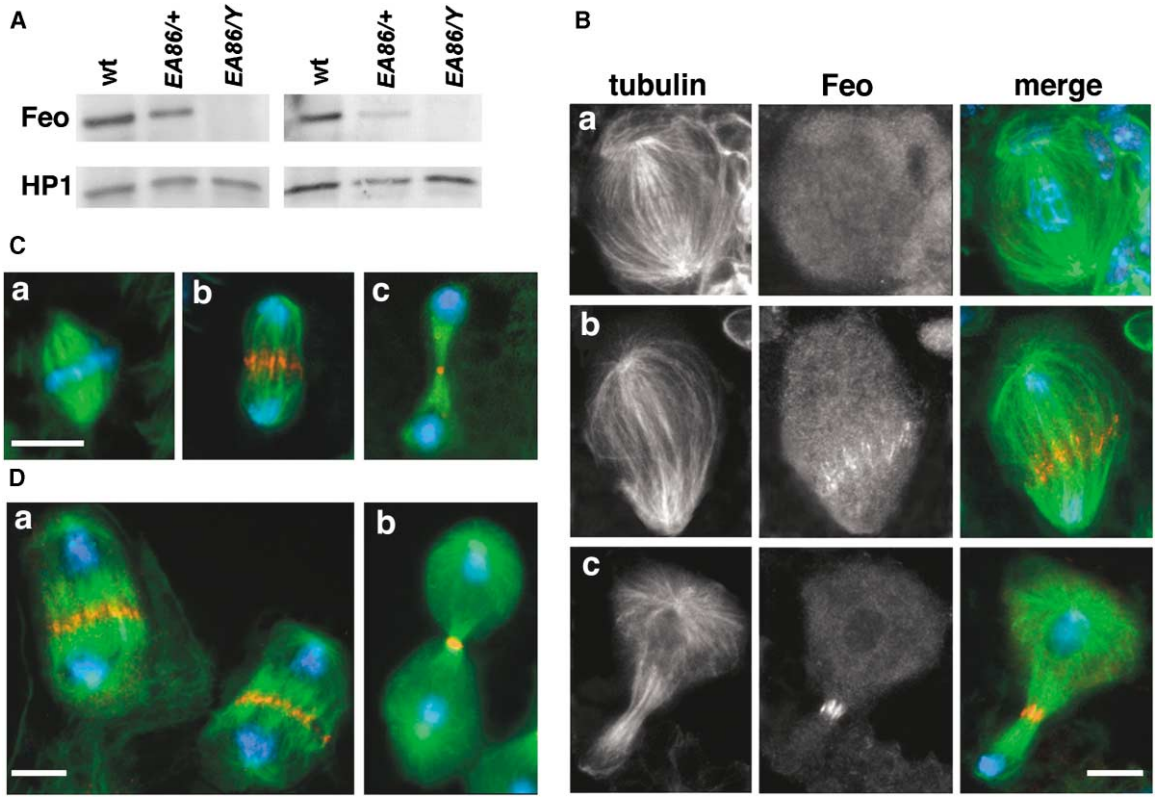


Figure 3. Subcellular Localization of the Feo Protein

(A) Western blots showing reduction and absence of Feo in $feo^{EA86}/+$ heterozygous females and $feo^{EA86}$ males, respectively. The two panels refer to the anti-$Feo^{1-148}$ (N-term) and anti-$Feo^{424-672}$ (C-term) antibodies. HP1 was used as a loading control. The truncated form of Feo encoded by $feo^{EA86}$ was not observed in mutant animals, suggesting that either the mutant transcript or the truncated protein is unstable.

(B) Wild-type neuroblasts stained for tubulin (green), Feo (orange), and DNA (blue); (a) early anaphase; (b) late anaphase; and (c) telophase.

(C) Wild-type ganglion mother cells stained for tubulin (green), Feo (orange), and DNA (blue); (a) metaphase; (b) late anaphase; and (c) telophase.

(D) Wild-type primary spermatocytes stained for tubulin (green), Feo (orange), and DNA (blue); (a) (right), late anaphase; (a) (left) early telophase and (b) late telophase. Feo accumulates at the central spindle midzone in all cell types.

The scale bars represent 5 μm.

dle that displays a broad Pav localization at the midzone and exhibit either a diffuse Asp signal or none at all. Based on these results, we suggest a model for Feo function. We propose that Feo interacts with the antiparallel MTs of the central-spindle midzone, so as to maintain their overlap during MT elongation and antiparallel sliding. In the absence of Feo, the plus ends of central-spindle MTs would either grow or move past the central-spindle midline and penetrate into the opposite half of the spindles. This would lead to telophase central spindles with improperly oriented MTs and a more diffuse localization of Asp and Pav (Figure S5).

The phenotype of Feo-depleted telophases is unique and differs markedly from that observed in S2 cells depleted of other proteins required for both central-spindle and contractile-ring formation. In the absence of Pav, Rho1, RacGAP50C, Pbl (a Rho GEF), or Sqh (a regulatory myosin light chain), S2 cells undergo anaphase A normally but then fail to form a central spindle and to undergo anaphase B, giving rise to telophases that are substantially shorter than their control counterparts [21]. These results indicate that Pav, Rho1, RacGAP50C, Pbl, and Sqh are directly or indirectly required for the stabilization of the central-spindle MTs and strongly suggest that plus-end elongation and MT sliding within the central spindle mediate anaphase B in S2 cells. In contrast, anaphase B does occur in *feo* RNAi cells, suggesting that the aberrant central spindles of Feo-depleted cells retain the ability to power spindle pole separation during anaphase.

The phenotype of Feo-depleted cells provides novel insight into the relationship between the central spindle and the contractile ring. Brain cells of *feo* mutants and Feo-depleted S2 cells form regular actin-enriched equatorial structures during late anaphase, but these structures are unstable and unable to constrict and mediate cytokinesis. In contrast, S2 cells depleted of Pav, Rho1, RacGAP50C, Pbl, or Sqh fail to assemble actin equatorial bands during late anaphase [21]. These results indicate that the transient interdigitation of antiparallel MTs observed at the center of Feo-depleted late anaphases is sufficient to stimulate the initial formation of a contractile apparatus. However, in the absence of a regular central spindle during telophase, the contractile ring is compromised. This suggests that molecules critical for the function of contractile rings continually reach the cleavage area during telophase. Such molecules most likely move to the central spindle midzone through the

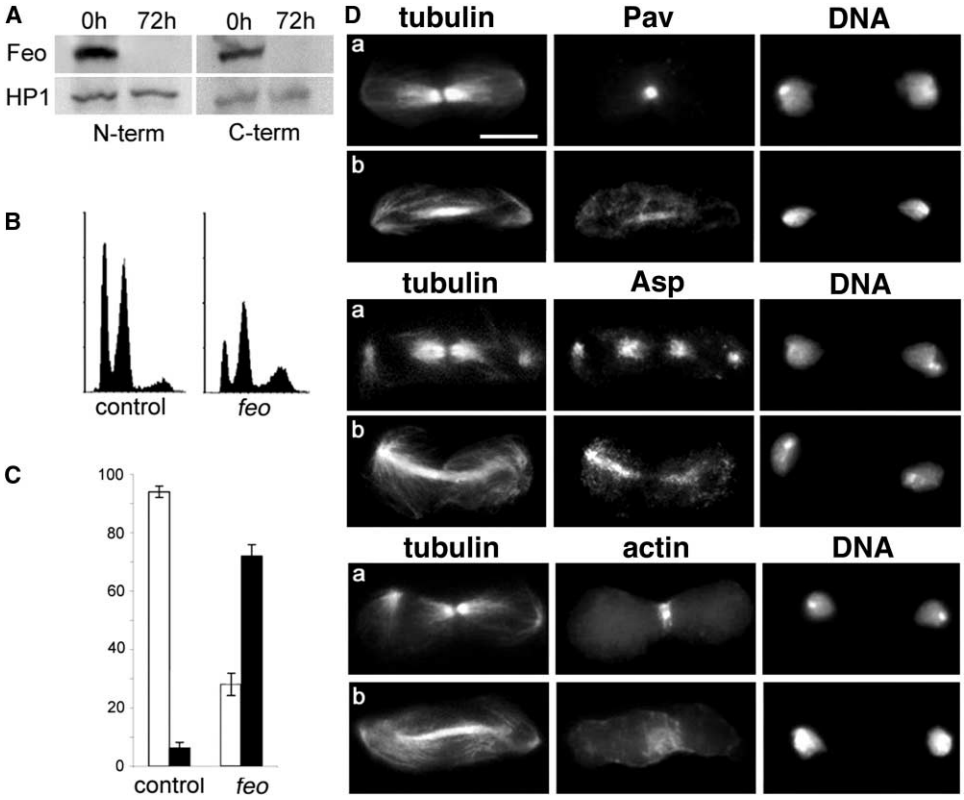


Figure 4. Depletion of Feo by RNAi Disrupts Central Spindle Organization and Causes Failures in S2 Cell Cytokinesis

(A) Western blots showing that Feo is no longer detectable after 72 hr treatment with *feo* dsRNA. The two panels refer to the anti-Feo$^{1-148}$ (N-term) and anti-Feo$^{424-672}$ (C-term) antibodies; HP1 was used as loading control.

(B) FACS profiles of control and *feo* RNAi cells showing that RNAi substantially increases the frequency of polyploid (8C) cells.

(C) Frequency of binucleated (filled columns) and mononucleated (empty columns) cells in control (n = 1388) and *feo* RNAi (n = 1231) cultures; error bars are 3× standard error.

(D) Cytological phenotypes of control and *feo* RNAi telophases stained for tubulin and DNA as well as Pav, Asp, or actin. (a) control telophases; (b) *feo* RNAi telophases. Note the peculiar organization of the central spindle and the absence of the actin ring in Feo-depleted telophases. The scale bar represents 10 μm.

action of plus end-directed motors and cannot reach the equator of Feo-depleted cells because of the disorganization of their central spindles.

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